TGFβ-signaling regulates the proliferation of Müller Glia-derived Progenitor Cells in the retina

Honors Research Thesis

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By

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Abstract:

Müller glia cells in the retina can be stimulated to form Müller glia-derived progenitor cells (MGPCs) that can regenerate neurons. Müller glia are typically the support cells of the retina, but upon treatment with NMDA damage or consecutive doses of FGF2 may be stimulated to de-differentiate and form proliferating progenitor cells. The cell signaling pathways involved in the formation of MGPCs are beginning to be revealed, and a better understanding will help to develop novel strategies for treating retinal diseases in humans. This study aims to investigate the role of TGFβ-signaling in the proliferation and formation of MGPCs in the avian retina. The results of this study indicate that TGFβ signaling components are expressed in the retina. Immunohistochemistry shows that the transcription factor smad2 is localized to Müller glia nuclei in untreated retinas, but following NMDA and FGF2-treatment expression in the cytoplasm is increased. This suggests a decrease in TGFβ-signaling when MGPCs are known to form. We find that treatment with recombinant TGFβ2 reduces the formation of MGPCs in damaged retinas, as well as expression of stem cell markers Pax6, Klf4, and Egr1. Conversely, applying small molecule inhibitors to the pathway significantly increased the number of proliferating MGPCs in both damage and undamaged retinas. Consistent with these findings, inhibition of TGFβ-signaling also increased proliferation at the Ciliary Marginal Zone (CMZ), a separate progenitor cell population at the peripheral edge of the retina. We conclude that TGFβ is yet another pathway that influences the reprogramming of Müller glia into proliferating MGPCs.

Methods and Materials:

Animals:

The use of animals in this study was in accordance with the guidelines set by the National Institutes of Health and the Ohio State University. Newly hatched leghorn chickens (*Gallus gallus domesticus*) were obtained from Meyer Hatchery in Polk, Ohio. Chicks were housed in a brooder around 25^{o}C and received water and Purina^{tm} chick starter *ad libitum*. Chicks were kept on a cycle of 12-hours light and 12-hours dark with lights on at 8:00 am.

Intraocular injections:

Chickens were anesthetized by inhalation of 2.5% isoflurane in oxygen. Intraocular injections were carried out as described previously (Fischer et al., 1998). In all experiments, the right eyes of chicks were injected with the "test" compound and the contra-lateral left eyes were injected with the control. Compounds were injected in sterile saline with 0.05 mg/ml bovine serum albumin added up to 20 μl. Compounds included NMDA (38.5 or 154 μg/dose), FGF2 (250 ng/dose; R&D systems), recombinant human TGFβ2 (300ng/dose; R&D Systems), Smad3 inhibitor SIS3 (4μg/dose; Sigma-Aldrich), and TGFβ-receptor (Alk5) inhibitor SB4315432 (2μg/dose; R&D Systems). EdU (2μg/dose) was added in order to label proliferating cells. Injection paradigms can be found in each figure.

Quantitative Reverse Transcriptase PCR:

RNA was isolated from Individual retinas placed in 1 ml of Trizol Reagent (Invitrogen). Isolation was performed according to the Trizol protocol and total RNA was re-suspended in

50µl RNAse free water. Genomic DNA was removed with the *DNA FREE* kit (Ambion). cDNA was synthesized from mRNA by using Superscript^{tm} III First Strand Synthesis System (Invitrogen) and oligodT primers according to the manufacturer's protocol. Reverse Transcriptase was excluded in control reactions to verify that primers did not amplify genomic DNA.

PCR primers were designed with the NCBI Primer-BLAST design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Sequences and predicted product sizes can be found in table 1. Standard protocols, Platinum^{tm}Taq (Invitrogen) and an Eppendorf thermal cycler were used to carry out PCR reactions. Products were run on an agarose gel to verify the predicted product sizes.

Fixation, sectioning and immunocytochemistry

Tissues were fixed, sectioned, and immunolabeled as described previously (Fischer, Ritchey et al. 2008). Working dilutions and sources of antibodies used in this study are listed in table 2. Secondary antibodies included donkey-anti-goat-Alexa488/568, goat-anti-rabbit-Alexa488/568, goat-anti-mouse-Alexa488/568/647 (Life Technologies) diluted to 1:1000 in PBS plus 0.2% Triton X-100.

EdU-Labeling:

EdU-labeling was performed on immunolabeled sections fixed in 4% formaldehyde in PBS for 5 min, washed in PBS for 5 min, permeabilized with 0.5% Triton X-100 in PBS for 1 min, and washed twice for 5 min in PBS, all carried out at room temperature. Sections were incubated at room temperature for 30 min in 2M Tris, 50 mM $CuSO_4$, Alexa Fluor 568 or 594

Azide (Thermo Fisher Scientific), and 0.5M ascorbic acid in dH_2O. Sections were washed with PBS for 5 minutes and prepared for microscopy.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL):

Cell death was detected using the TUNEL method to identify cells that contained fragmented DNA. An *In Situ* Cell Death Kit (TMR red; Roche Applied Science) was used as per the manufacturer's instructions.

Photography, measurements, cell counts and statistics:

Images were collected using a Leica DM5000B microscope equipped with epifluorescence and Leica DC500 digital camera. Confocal images were obtained using a Leica SP8 imaging system at the Hunt-Curtis Imaging Facility at The Ohio State University. Adobe Photoshop was used to optimize images and build figures. For each experiment, cell counts were performed on representative images from consistent regions of the retina, to avoid region-specific variability. Immunofluorescence was quantified using ImagePro6.2 (Media Cybernetics, Bethesda, MD, USA) on retinal regions extracted from 5.4 MP digital images, similar to previous reports (Fischer, Scott et al. 2009). Images were obtained from identical camera and microscope settings for each data set.

Image samples containing nuclei of bipolar and amacrine cells in the inner nuclear layer were randomly selected for further analyses. Measurement for content in the nuclei of Müller glia/MGPCs were made by selecting the total area of pixel values ≥70 for Sox2 or Sox9 immunofluorescence (in the red channel), and copying nuclear Smad2/3, KLF4, or Pax6 (in the

green channel). This copied data was pasted into a separate file for quantification or onto 70% grayscale background for figures. Measurements were made for regions containing pixels with intensity values of 68 or greater (0 = black and 255 = saturated); a threshold that included labeling of inner retinal neurons. The total area was calculated for regions within defined threshold pixel intensities and average pixel intensity was calculated, as well as the total pixel value. These calculations were made for retinal regions obtained from a minimum of six different retinas for each experiment.

In Figures 1c-e, the percentage of Smad2 in Sox2+ nuclei in the INL was determined as follows. Fixed areas of INL were cropped and ImagePro 6.02 was used to measure immunofluorescence as the summation of pixel values (density sum) above threshold (68 in the green channel) in each cropped region of retina. Then the area, within that region, occupied by Sox2-labeling was selected in the red channel (pixel value of 180 ± 75) and the same selected area was cut and pasted from the green channel (Smad2) into a separate image for quantification. For each individual image (n=6) the percentage of Smad2 (above threshold) present in Sox2-labeled nuclei over the total density sum of Smad2 within cropped regions of the INL was calculated and averaged for control and NMDA-treated retinas.

For proliferation and cell counts, central retina was defined as the region within a 3mm radius of the posterior pole of the eye, and peripheral retina was defined as an annular region between 3mm and 0.5mm from the CMZ. EdU-labeled cells were identified on the basis that 100% of proliferating cells in the chick retina are comprised of Sox2/9^{+} Müller glia in the INL/ONL, Sox2/9/Nkx2.2^{+} Non-Astrocytic Inner Retinal Glia (NIRG) cells in the IPL, GCL and NFL, and CD45^{+} (Sox2/9^{-}) microglia (Fischer, Scott et al. 2010). Sox2^{+} nuclei of Müller glia in the INL

are noticeably different from the $Sox2^+$ nuclei of cholinergic amacrine cells which are small and round (Fischer, Scott et al. 2010).

GraphPad Prism 6 was used for statistical analyses. We performed a two-tailed, paired t-test to determine significance of difference between treatment groups when taking into consideration inter-individual variability (treated-control). A two-tailed, unpaired t-test was used to determine significance of difference between two treatment groups.

Introduction:

Age-related macular degeneration, retinitis pigmentosa, and other diseases of the retina result in death of retinal neurons and irreversible vision loss. Potential treatments include therapies to prevent neuronal cell death, or replace those that are lost. The latter option may be feasible by harnessing the regenerative potential of Müller glia. Müller glia are normally implicated in maintaining structural and synaptic support within the retina; however, these cells can be stimulated to re-enter the cell cycle and proliferate as Müller glia-derived progenitor cells (MGPCs) (Reichenbach et al., 2013). In fish and bird, this reprogramming involves the de-differentiation of Müller glia and up-regulation of retinal stem cell transcription factors such as Pax6, Klf4, Ascl1a, and Chx10 (Gallina et al. 2014a; Goldman, 2014) Additionally, Müller glia in the normal mammalian retina also maintain transcriptional profiles that overlap with retinal progenitor cells (Blackshaw et al., 2004). Therefore, MGPCs have the potential to differentiate into retinal neurons and replace those lost with damage; however, the regenerative capacity of the retina is considerably different between vertebrate classes. Zebrafish have the ability to regenerate a fully functional retina following damage (Lenkowski et

al, 2013). On the other hand, mammals typically undergo reactive gliosis in response to damage, and a very limited number of Müller glia may become neurogenic (Karl and Reh, 2010). The following studies were completed in the chick model system, which provides an intermediate capacity for regeneration. *In vivo*, many Müller glia in the chick retina can be stimulated to undergo a single round of division (Fischer and Reh, 2001). Understanding the network of signaling pathways involved in Müller glia reprogramming across vertebrate classes is crucial to harnessing the regenerative capacity of retina and MGPC-mediated regeneration as a potential therapy in higher vertebrates.

The Transforming Growth Factor-β (TGFβ) signaling pathway is another pathway that has been shown to be active in this network. TGFβ-signaling has been shown to regulate MGPC formation in zebrafish and rodents (Close et al., 2005; Lenkowski et al., 2013.) TGFβ superfamily members signal through a complex of type I and type II serine/threonine kinase receptors, allowing potential for a diversity of combinations and outputs (Wu and Hill, 2009). Activation of TGFβ heteromeric complexes leads to phosphorylation of isoforms of Smad proteins, specifically Smads 2/3. Receptor regulated Smads (R-smads) form a complex with Smad4 for translocation to the nucleus and regulation of transcription (Guo and Wang, 2009). Though the response of Smad proteins and complexes in this signaling cascade has been identified, their influence on cellular phenotypes is not well understood. Inhibitory Smads have also been identified in the retina, however, their impact on MGPC formation is poorly understood. One study indicated no change in Müller glia reactivity or proliferation with heterozygote loss of function of inhibitory smad7 (Kugler et al., 2015). Therefore, further studies are necessary to understand the role of TGFβ-signaling and Smad proteins in normal and damaged retinas.

Accordingly, the purpose of these studies was to investigate how TGFβ-signaling influences Müller glia reprogramming in the chick retina.

Results:

Retinal Damage influences TGFβ-signaling in M*ü*ller Glia

qRT-PCR was used to quantify mRNA levels of TGFβ ligands in the normal retina and 48 hours following NMDA damage when MGPCs are known to form (Fischer and Reh 2001). We found that *tfgb1, tgfb2,* and *tgfb3* were all expressed in the retina, however, levels did not noticeably change in response retinal damage (Fig. 1a). The levels of *tgfb2* suggested the highest expression, consistent with previous reports that *tgfb2* is expressed throughout the brain and retina of rodents (Close et al. 2005; Constam et al. 1994). To confirm that TGFβ-signaling is active in normal and damaged retinas, retinal sections were immunolabeled for the TGFβ-associated transcription factor Smad2. Antibodies to psmad2 failed, therefore we used patterns and localization of Smad2 as a readout of signaling through TGFβ. Antibodies to Smad2 would recognize both the active, phosphorylated form in the nucleus, as well as the inactive form in the cytoplasm. Therefore, localization to Müller glia nuclei and co-localization with the nuclear marker sox2 indicated active TGFβ-signaling in control retinas(Fig. 1d-e). In response to retinal damage, Smad2 was instead distributed throughout the cytoplasm. The presence of Smad2 in the cytoplasm indicated that TGFβ-signaling was inactive at the time of fixation, but cells still had the capacity to respond to TGFβ-signaling. In the untreated retina, 60% of Smad2-immunofluorescnece was found in Müller glia whereas, only 20% co-localized with Sox2+ Müller glia at 72 hours after damage (Fig. 1b-e). This suggests that TGFβ-signaling through

transcription factor Smad2 is downregulated in response to retinal damage, at a time that MGPCs are known to form.

TGFβ-signaling attenuates Müller glia proliferation in the NMDA-damaged chick retina

Intraocular injections were performed to influence TGFβ-signaling components and assess its role in the formation of MGPCs in the chick retina. TGFβ-signaling pathway was stimulated with recombinant TGFβ2, the TGFβ-ligand most abundantly expressed in the chick retina (see Fig 1a). We found that activation of TGFβ-signaling following retinal injury substantially reduced the formation of MGPCS (Fig. 2a,b). TGFβ2 also inhibited the proliferation of microglia but did not influence non-astrocytic inner retinal glia (NIRGs) (Fig. 2c,d). Retinas treated with TGFβ2 also showed a reduction in levels of the retinal stem cell factors Pax6 and Klf4 (Fig. 2e-h). Pax6 is a transcription factor required in retinal development and plays a role in retinal progenitor cell proliferation (Thummel et al, 2010). Additionally, we found that Egr1 was downregulated in Müller glia and bipolar cells following activation of TGFβ-signaling (Fig. 2i). Egr1 is an immediate early gene associated with MAPK-signaling, a pathway known to stimulate Müller glia proliferation (Fischer et al. 2009b).

Because activation of TGFβ signaling suppressed Müller glia reprogramming, we investigated whether inhibition of the pathway stimulates the formation of proliferating MGPCs. We performed intraocular injections of SIS3, which inhibits the interactions of TGFβ-associated transcription factor Smad3 with Smad4 and subsequent translocation of the Smad complex to the nucleus (Jinnin et al. 2006). Inhibition of TGFβ-signaling following retinal damage resulted in a significant increase in the formation MGPCs (Fig. 3 a-c). Inhibition only

affected proliferation of MGPCs, because numbers of proliferating microglia and NIRGS remained unchanged (Fig. 3 b).

To confirm the effects of inhibition of TGFβ-signaling, we targeted additional components of the TGFβ-pathway. Intraocular injections of small molecule inhibitor SB4321542 (SB-43) were applied to block TGFβ type receptor 1 (TGFβ-R1) and the activin receptor like kinases (ALK) (Inman et al. 2002). Suppression of TGFB-R1 and ALK also significantly increased MGPC formation following NMDA-treatment and similar to SIS3 inhibition, proliferation of microglia and NIRG cells was unaffected (Fig. 3c). Inhibition of TGFβ-signaling also failed to alter levels of Pax6 or Klf4 (Fig. 3d,g). However, inhibition with SB-43 did appear to influence mTOR-signaling and resulted in increased expression of mTOR read-out pS6 (Fig. 3e,f) This suggests that TGFβ-signaling may also influence mTor-signlaing in the retina.

Inhibition of TGFβ-signaling in augments MGPC formation in FGF-treated retina

Because inhibition of TGFβ-signaling stimulated MGPC formation following NMDA-treatment, we investigated whether TGFβ-signaling influences proliferation in the absence of damage. We found that application of SIS3 alone did not influence Müller glia in normal retinas (data not shown). However, four consecutive daily doses of FGF2 has been shown to stimulate Müller glia proliferation in the absence of damage (Fischer et al., 2014b). Therefore, we tested whether inhibition of TGFβ-signaling would stimulate FGF2/MAPK signaling and supplement FGF2-mediated MGPC formation. Co-application of FGF2 and SIS3 for two days increased levels of MAPK read-out cFos in Müller glia(Fig. 4a,b). Short term treatment did not appear to influence mTor signaling indicated by levels of ps6 (Fig4c,d). With four consecutive daily doses

of FGF2, TGFβ-signaling through transcription factor Smad2 appeared to decrease compared to saline treated controls (Fig. 4e). Similar to NMDA-treatment, this suggests that TGFβ-signaling is decreased in Müller glia when MGPC are known to form (See Fig. 1a). Additionally, co-application of SIS3 and FGF2 for 3 consecutive days significantly increased MGPC formation (Fig. 4f,g).

We were unable to determine the effect of TGFβ2 activation on FGF2-mediated MGPC formation. Co-application resulted in extensive retinal damage and significant amounts of TUNEL-positive cells were detected in the GCL and INL. Retinal cell death likely influenced MGPC cell counts, therefore this data was excluded. We were unable to determine the cause of damage, but blood within the vitreous humor indicated that vascular integrity may have been compromised. Some studies indicate that increased signaling and activity of TGFβ ligands in the eye may cause adverse inflammatory effects and potentially tissue fibrosis (Saika, 2006). Interestingly, the combination of FGF2 and IGF1 also results in cell death in the retina, while neither factor alone produces significant damage (Ritchey et al., 2012).

TGFβ-signaling influences the proliferation of retinal progenitors in the circumferential marginal zone (CMZ)

We next tested whether inhibition of Smad3 influences progenitor cell populations other than MGPCs, specifically retinal progenitors in the circumferential marginal zone (CMZ). At the peripheral edge of the retina, the CMZ represents a region of progenitor cells that persists throughout the life of the animal (Ghai et al., 2008; Lamba et al. 2008). While, SIS3 alone did not influence proliferation of CMZ progenitors (data not shown), co-application of

SIS3 synergized with insulin-like growth factor 1 (IGF1) to increase proliferation of CMZ progenitors. Intraocular injections of IGF1 alone is known to stimulate the proliferation of CMZ progenitors (Fischer and Reh, 2000). Co-application of SIS3 with IGF1 nearly doubled the number of newly generated cells added to the retina from proliferating CMZ progenitors (Fig. 5a-c). This suggests that TGFβ-signaling through Smad3 may suppress proliferation of CMZ progenitors that are responsive to IGF1, but does not affect CMZ progenitors that proliferate under normal conditions.

Discussion:

Altogether, our findings suggest that TGFβ-signaling inhibits the de-differentiation and proliferation of Müller glia as MGPCs. TGFβ-signaling contributes to a long list of cell-signaling pathways that play a role in the formation of MGPCs across vertebrate classes. In the avian retina, this network of pathways is known to include MAPK, mTor, Notch, and Glucocorticoid (Fischer et al.,2002; Zelinka et al, 2016; Ghai et al., 2010; Todd and Fischer, 2015; Gallina et al., 2014b).

We found TGFβ-signaling components, including ligands and Smad transcription factors, are present in the normal retina. This is consistent with reports that suggest TGFβ2 is expressed by inner retinal neurons and TGFβ receptors are expressed by both Muller glia and retinal progenitor cells (Close, 2005). Active Smad2 is decreased in Müller Glia at 2 and 3 days following damage, suggesting that TGFβ-signaling is downregulated at a time when MGPCs are known to form. Importantly, the nucleocytosolic shuttling of Smads has been well described in development, and is believed to accurately reflect levels of receptor activation (Wu and Hill,

2009). However, questions remain on how exactly TGFβ-signaling through Smad transcription factors influences gene expression and cellular phenotypes.

Additionally, we found that activation of TGFβ-signaling after damage suppressed proliferation of MGPCS and expression of stem cell markers associated with retinal progenitor cells. We saw a decrease in MAPK early gene, Egr1 in Müller glia. Interestingly, TGFβ-signaling has been shown to act in opposition of the MAPK pathway, where TGFβ-signaling may suppress proliferation (Chapnick, et al., 2011). Because MAPK is known to be a crucial pathway in the formation of MGPC, this provides further evidence that TGFβ-signaling may be antagonistic to Müller glia reprogramming in the retina (Fischer et al., 2009). Conversely, we observed an increase in Müller glia proliferation following inhibition of TGFβR-1 and Smad3 in damaged retinas. Inhibition of transcription factor Smad3 also augmented proliferation in undamaged retinas, with FGF2-mediated MGPC formation.

These results are consistent with previous studies in the zebrafish and rodent retina. In the zebrafish retina, the upregulation of repressors to TGFβ-signaling is necessary to drive Müller glia mediated regeneration following damage (Lenkowski et al. 2013). Studies in the rat retina suggests that inhibition of TGFβ may also stimulate MGPC formation in mammals (Close et al. 2005). Taken together, these data suggest that suppression of TGFβ-signaling is necessary to promote Müller glia reprogramming across vertebrate classes.

Lastly, Smad3 inhibition influenced retinal progenitor cells at the CMZ in a similar fashion. The CMZ of chicks maintains a progenitor cell population that continues to proliferate at the peripheral edge of the chick retina in early postnatal development. Although the activity of these cells decreases in the post-hatch chick retina, proliferation can be stimulated by factors

such as Insulin, IGF, and FGF (Fischer and Reh, 2003). Our results indicate that SIS3 in combination with IGF, but not SIS3 alone, was sufficient to increase proliferation at the CMZ. Many additional pathways and factors have been shown to influence the addition of cells at the CMZ of chicks. These results are similar to recent findings, where HB-EGF in combination with IGF, but not alone, influenced progenitor cells at the peripheral edge of the retina (Todd et al., 2015).

Collectively, these studies suggest that TGFβ-signaling plays a role in maintaining quiescent Müller glia and CMZ progenitors in the retina, and inhibits proliferation within these cell populations. Studies in development suggest that TGFβ-signaling components are available to act on late stage retinal progenitors during retinal histogenesis (Close et al., 2005). Additionally, Transforming Growth Interacting Factor (TGIF) acts as a repressor of TGFβ-signaling and may influence differentiation of progenitors in the developing retina (Satog and Watanabe, 2008). This suggests that TGFβ-signaling suppresses proliferation, but may also promote glial differentiation during retinal development, however, we failed to detect any changes in MGPC differentiation with inhibition of TGFβ-signaling following damage (data not shown).

The Bone Morphogenic Proteins (BMPs) and BMP-signaling pathway represent another component of the TGFβ superfamily. Furthermore, there is evidence to support that different sectors of this superfamily differentially influence MAPK and PI3K/AKT signaling. Our results suggest that MAPK read-outs were upregulated when TGFβ-signaling was suppressed, however other studies suggest mechanisms where TGFβ-signaling may induce p38 MAPK signaling (Sorrentino et al., 2008; Kawahara et al., 2008) Additional data suggests an antagonistic

relationship between BMP-signaling and TGFβ-signaling, which both act through Smad proteins and converge on co-Smad4 (Wu, 2009). Therefore, further studies on the interaction between these two pathways may provide a better understanding of their role in regulating Müller glia. We conclude that the TGFβ-signaling pathway regulates Müller glia de-differentiation and proliferation in the avian retina, and inhibition of this pathway is a promising approach to increasing the regenerative capacity of the retina.

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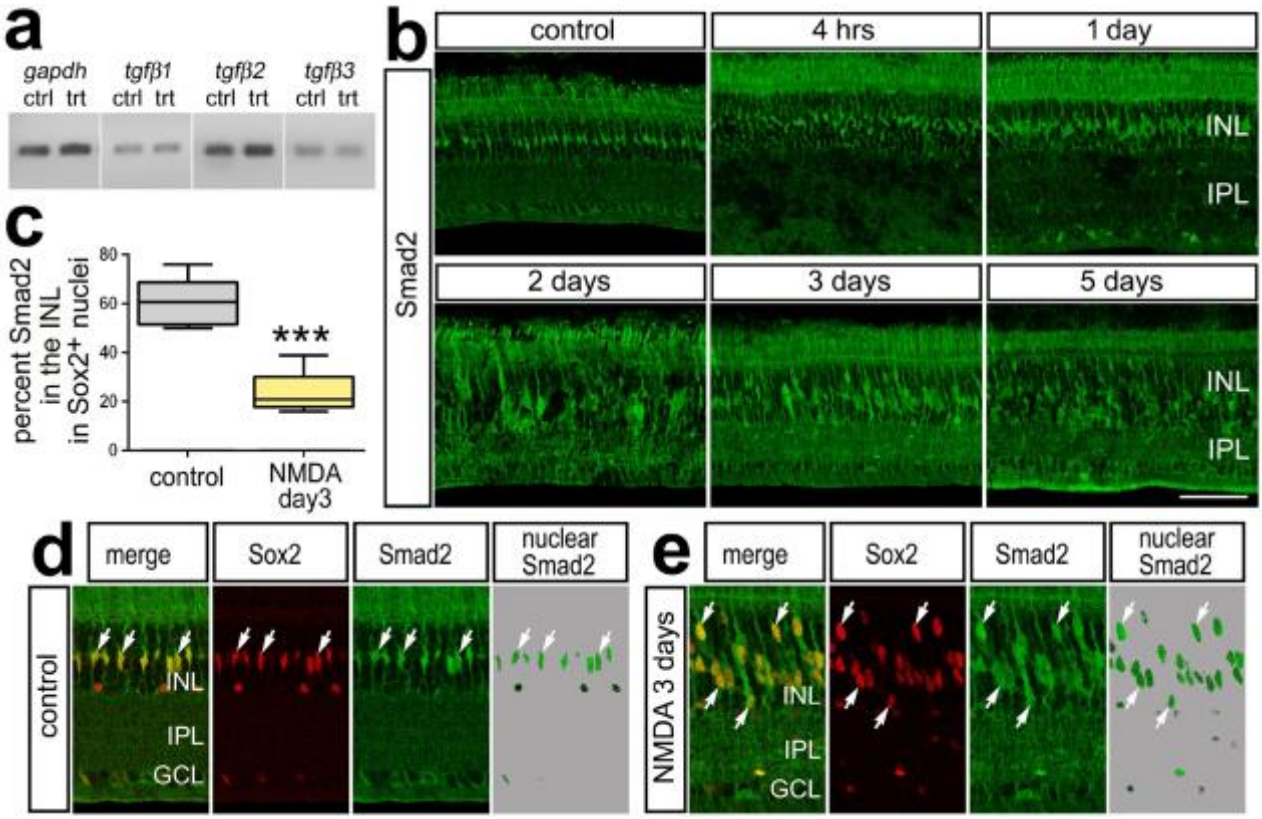

Figure 1: Retinal Damage influences TGFβ-signaling in Müller Glia and the Avian Retina

Retinas were obtained from eyes that received 500 nmol NMDA and harvested at different times after treatment. **a**; RT-PCR was used to detect *gapdh*, *tgfβ1*, *tgfβ*2 and *tgfβ3* in control and damaged retinas at 2 days following NMDA treatment. **b**; Sections were labeled with antibodies to Smad2 **c**: The mean (±SD; n=6) percentage of Smad2 in INL that overlaps with $Sox2^+$ nuclei of Müller glia. **d** and **e**: Confocal optical sections of control and NMDA (day 3) – treated retinas demonstrating the overlap of Smad2 in $Sox2^+$ nuclei of Müller glia or MGPCs. A two-tailed paired student's t-test was used to determine significance of difference (***$p<0.001$)

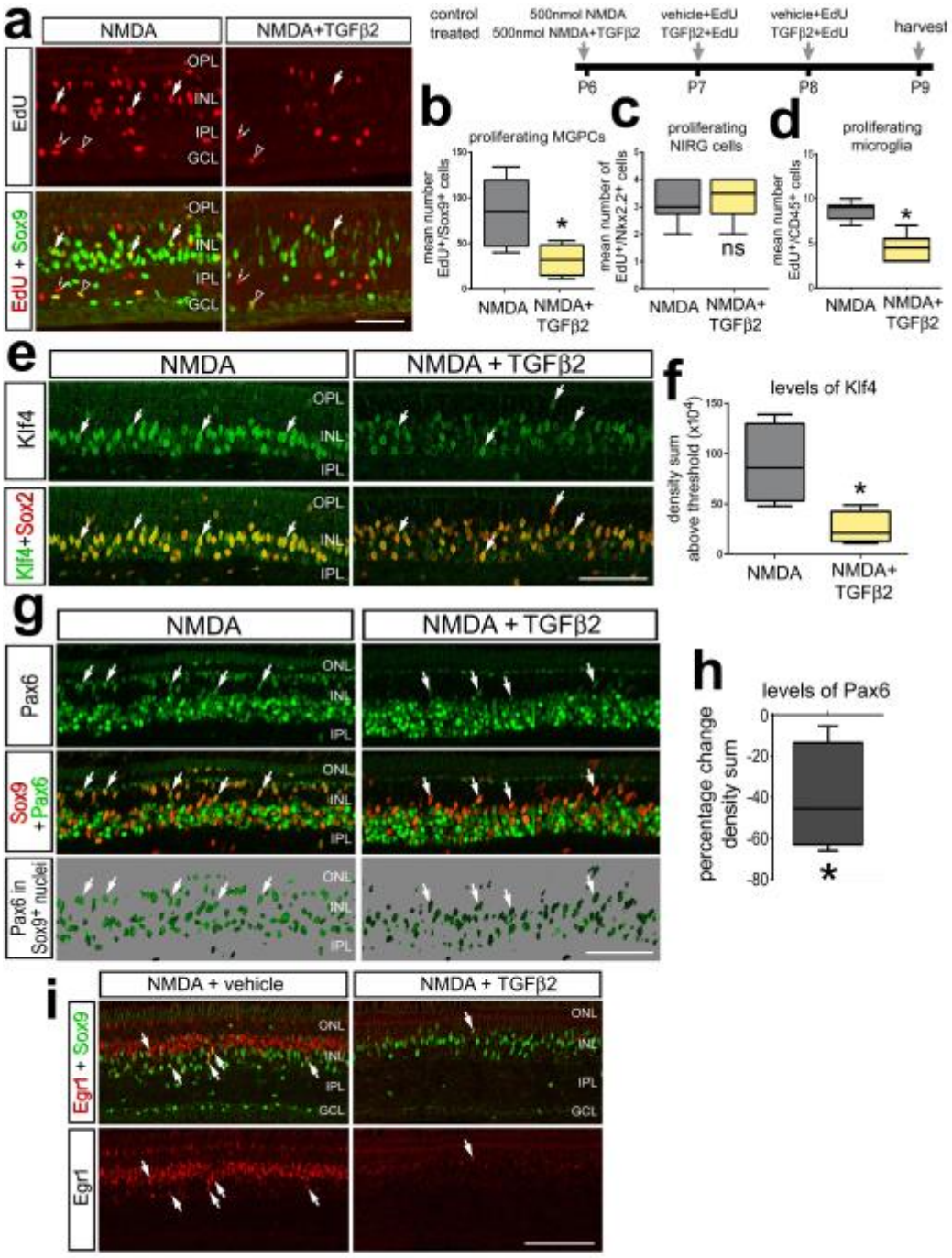

Figure 2: ***TGFβ-signaling attenuates Müller glia proliferation in the NMDA-damaged chick retina***

Retinas were obtained from eyes that received 500 nmol NMDA ± TGFβ2 at P6, EdU ± TGFβ2 at P7 and P8, and harvested at P9. Sections were labeled for EdU (red) and antibodies to Sox9 (green; **a**), Klf4 (green) and Sox2 (red; **e**), Pax6 (green) and Sox9 (red; **g**), or Sox9 (green) and Egr1 (red; **i**). The box plots illustrate the mean (±SD, n=7). The plots illustrate the number of proliferating MGPCs (**b**), NIRG cells (**c**), and microglia (**d**), levels of Klf4 in the nuclei of Müller glia/MGPCs (**f**), and percent change in the nuclear levels of Pax6 in Müller glia/MGPCs (**h**). A two-tailed paired student's t-test was used to determine significance of difference (*p<0.05, ***p<0.001) Arrows indicate the nuclei of MGPCs. Abbreviations: INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer, ONL – outer nuclear layer.

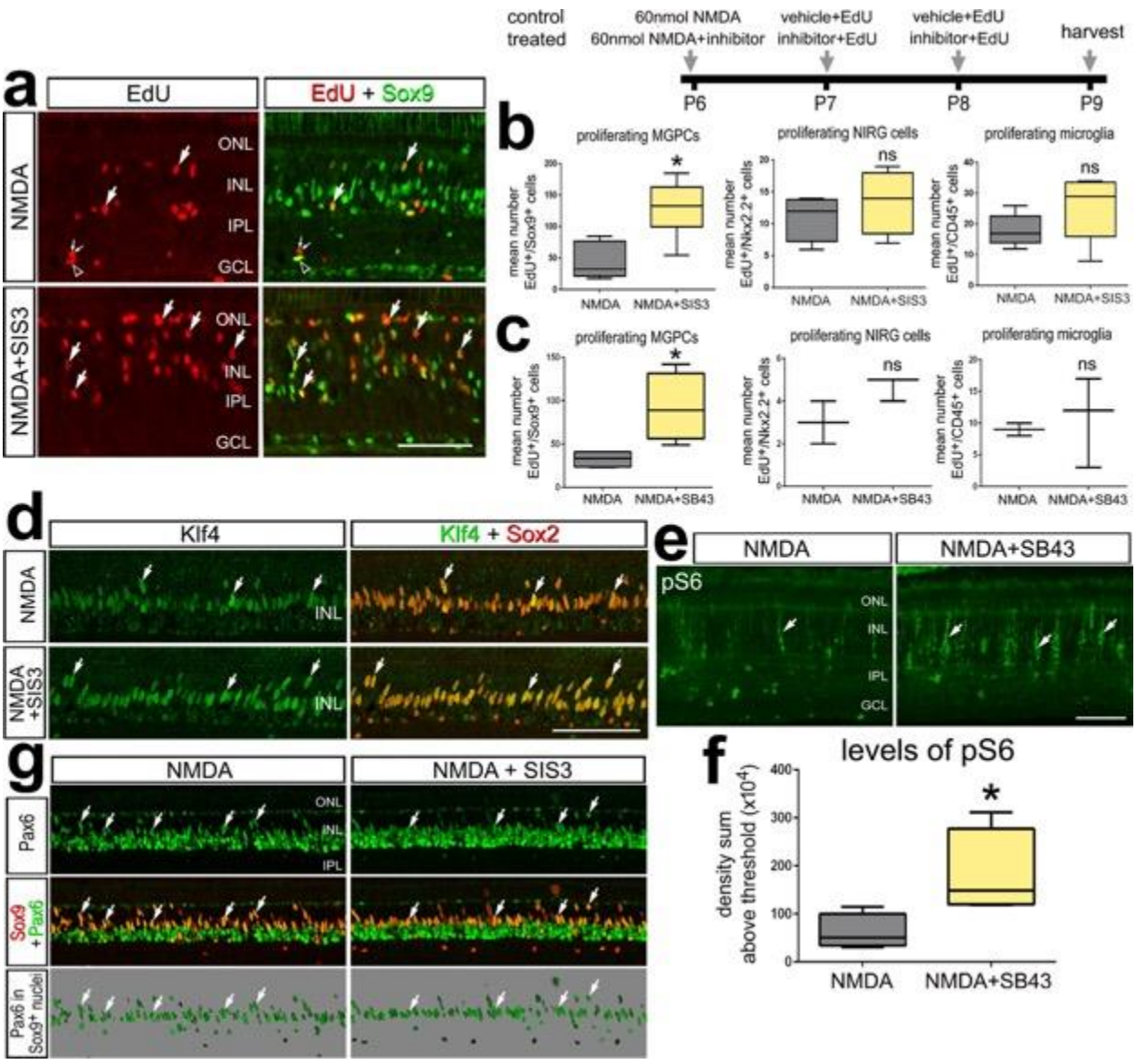

Figure 3. ***Inhibition of TGFβ-signaling stimulates MGPC formation in damaged retinas*** Eyes were Retinas were obtained from eyes that received 60 nmol NMDA ± inhibitor (SIS3 or SB431542) at P6, EdU ± inhibitor at P7 and P8, and harvested at P9. Sections were labeled for EdU (red) and antibodies to Sox9 (green; **a**), Klf4 (green) and Sox2 (red; **d**), pS6 (green; **e**), and Pax6 (green) and Sox9 (red; **g**).The box plots illustrate the mean (±SD, n>4). The plots illustrate changes in numbers of proliferating MGPCs, NIRG cells, and microglia (**b,c**), and levels of pS6 in Müller glia/MGPCs (**f**). A two-tailed paired student's t-test was used to determine significance of difference (*p<0.05, ***p<0.001). Arrows indicate the nuclei of MGPCs. The calibration bars in **a**, **d**, **e** and **g** indicate 50 µm. Abbreviations: INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer, ONL – outer nuclear layer.

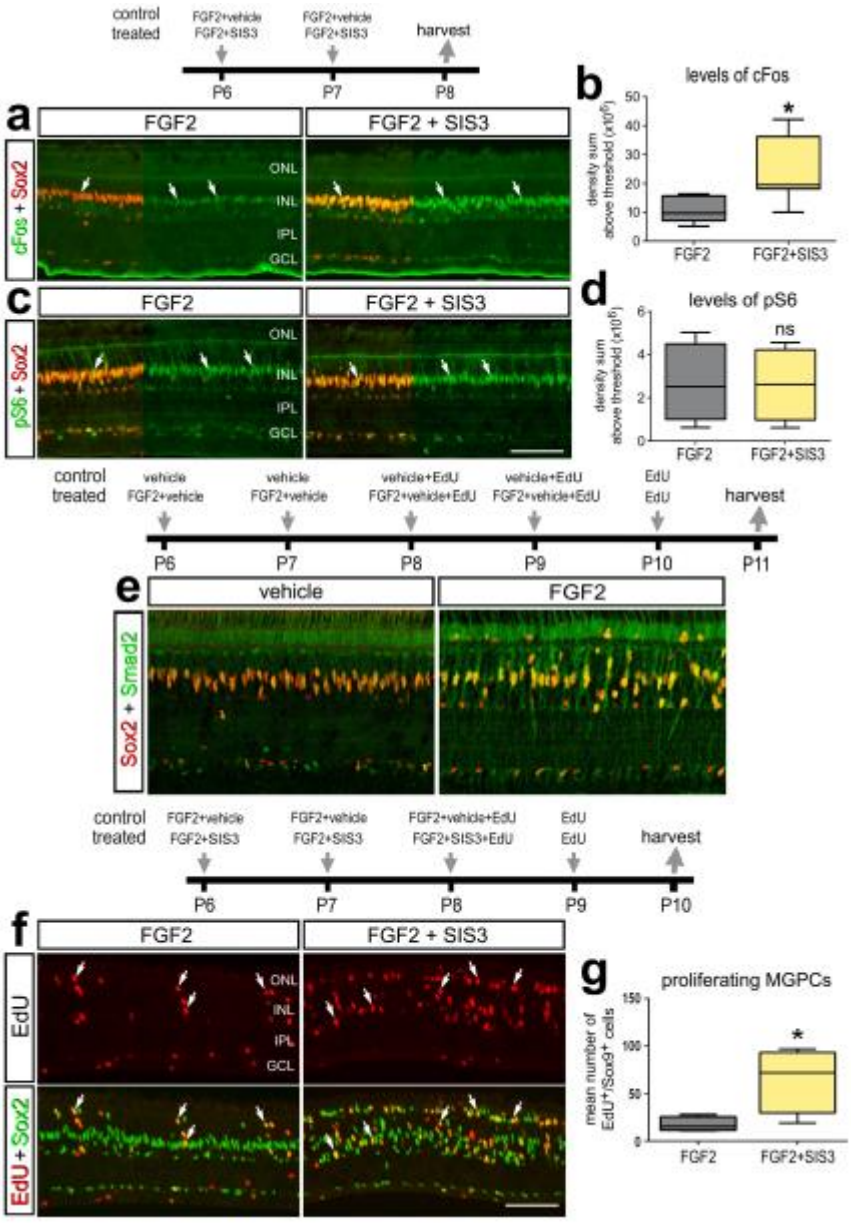

Figure 4. ***Inhibition of TGFβ-signaling in augments MGPC formation in FGF-treated retina***

Retinas were obtained from eyes that received FGF2±SIS3 at P6 and P7 **(a-d);** saline (control) or FGF2 at P6-P10 **(e);** or FGF2±SIS3 at P6, P7 and P8, EdU at P9 **(f-g);** and harvested one day later. Sections of the retina were labeled with antibodies to cFos (green) and Sox2 (red) (**a**), ps6 (green) and Sox2 (red) (**b**), Smad2 (green) and Sox2 (red) (**e**), and EdU (red) and Sox2 (green) (**f**). The box plots illustrate the mean (± SD, n>4) The plots illustrate levels (intensity sum) of cFos (**b**) and levels of pS6 (**d**). The plot in (**g**) represents the number of proliferating MGPCs (sox2+/EdU+ cells). A two-tailed paired student's t-test was used to determine significance of difference (*p<0.05, ***p<0.001). Arrows indicate the nuclei of MGPCs. Abbreviations: INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer, ONL – outer nuclear layer.

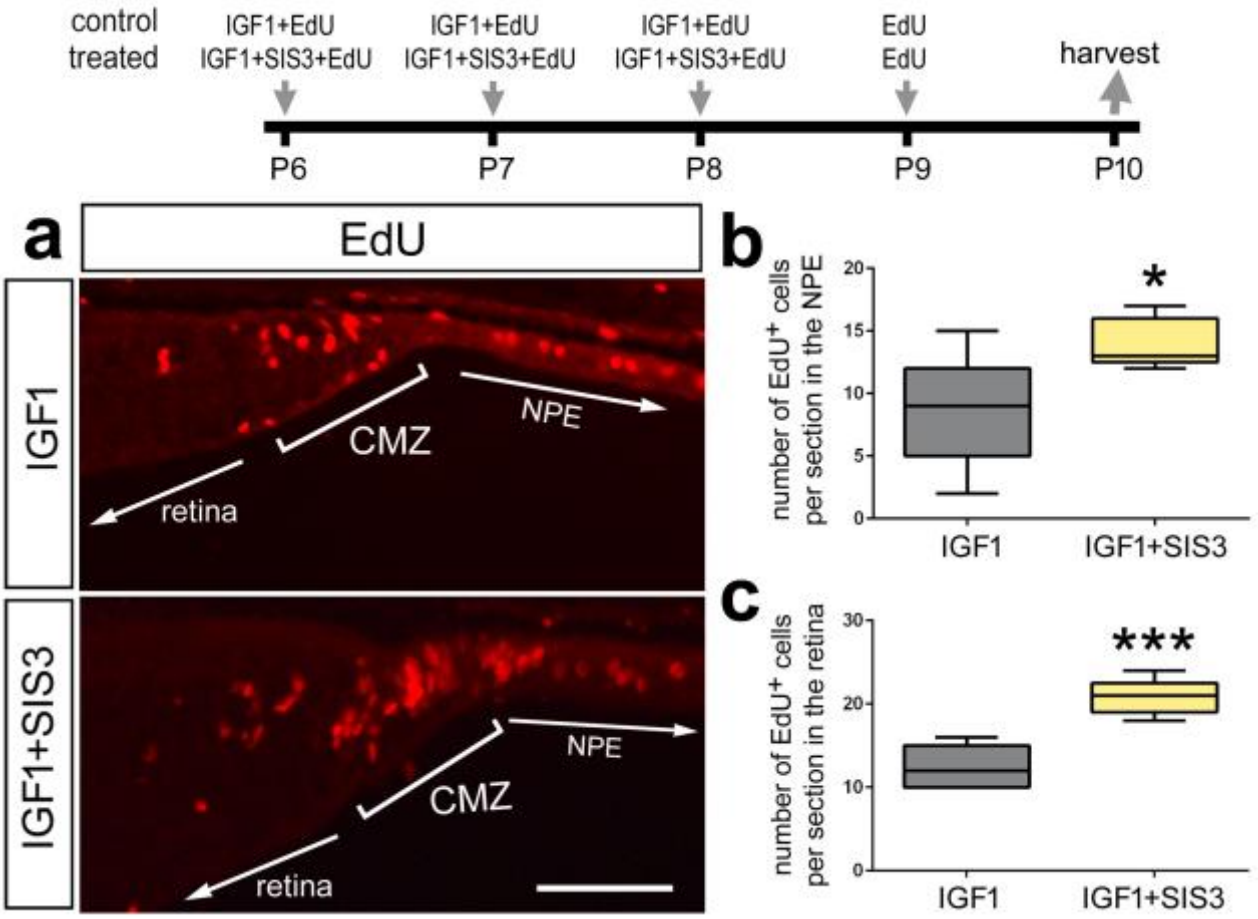

Figure 5: ***TGFβ-signaling influences the proliferation of retinal progenitors in the circumferential marginal zone (CMZ)*** Retinas were obtained from eyes that received 3 consecutive daily injections of IGF and EdU ± SIS3 (P6,P7,P8) followed by EdU alone (P9). Sections of the retina were labeled for EdU (red; **a**). The box plots illustrate the mean (±SD; n=5) numbers of proliferating cells in the non-pigmented epithelium (**b**) and retinal margin (CMZ + retina; **c**) A two-tailed paired student's t-test was used to determine significance of difference (*p<0.05, ***p<0.001). Abbreviations: CMZ – ciliary marginal zone, NPE - Non-pigmented epithelium.